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Genetic Analysis of the Mobilization and Leading Regions of the IncN plasmids pKM101 and pCU1

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The conjugative IncN plasmids pKM101 and pCU1 have previously been shown to contain identical *oriT* sequences as well as conserved restriction endonuclease cleavage patterns within their *tra* regions. Complementation analysis and sequence data presented here indicate that these two plasmids encode essentially identical conjugal DNA-processing proteins. This region contains three genes, *traI*, *traJ*, and *traK*, transcribed in the same orientation from a promoter that probably lies within or near the conjugal transfer origin (*oriT*). Three corresponding proteins were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and complementation analysis confirmed that this region contains three *tra* complementation groups. All three proteins resemble proteins of the IncW plasmid R388 and other plasmids thought to have roles in processing of plasmid DNA during conjugation. The hydropathy profile of TraJ suggests a transmembrane topology similar to that of several homologous proteins. Both *traK* and *traI* were required for efficient interplasmid site-specific recombination at *oriT*, while *traJ* was not required. The leading region of pKM101 contains three genes (*stbA*, *stbB*, and *stbC*), null mutations in which cause elevated levels of plasmid instability. Plasmid instability was observed only in hosts that are proficient in interplasmid recombination, suggesting that this recombination can potentially lead to plasmid loss and that Stb proteins somehow overcome this, possibly via site-specific multimer resolution.

Studies on the conjugal transfer (*tra*) systems of several plasmids in gram-negative bacteria, including IncF, IncP, IncW, IncQ, IncI, and IncX plasmids, have demonstrated that these systems have functional similarities and extensive sequence similarities at the DNA and protein levels (reviewed in references 32 and 67). This family of DNA transfer systems also includes the virulence (*vir*) regulon carried by Ti (tumor-inducing) plasmids of *Agrobacterium* spp., which are responsible for the transfer of tumorigenic DNA to higher plants (75).

Conjugal DNA processing in these plasmids requires plasmid-encoded proteins that interact with a small DNA sequence known as the origin of transfer (*oriT*) to introduce a strand-specific cleavage at a unique site referred to as the *nic* site (25, 42, 44, 58, 65). Following cleavage, one protein remains covalently bound to the 5′ end of the cleaved strand, and this strand is unwound in the 5′-to-3′ direction (45). According to a widely held model, complementary-strand synthesis is initiated from the free 3′ end of the cleaved strand in a manner resembling rolling-circle replication (14). In this model, a second cleavage is introduced into the reconstituted *oriT* after unwinding, and the enzyme ligates the 3′ and 5′ ends of the unwound DNA (46). The mechanism by which the processed strand is transferred into the recipient cell is as yet unknown, but transfer and unwinding are believed to occur simultaneously (32).

In the IncF, IncW, IncP, IncI, and IncQ conjugative systems and the *Agrobacterium vir* system, cleavage at *oriT* requires the action of two proteins, the smaller of which binds to the *oriT* in a plasmid-specific manner (15, 39, 43, 44). It is believed that binding of the smaller protein is required for recognition of the *nic* site by the second, larger protein, which cleaves and reseals the *oriT*. Such enzymes are often designated relaxases. Three conserved sequence motifs have been identified within these relaxase domains, suggesting that they may all have a common ancestry (2, 47). In the IncF and IncW proteins, the C-terminal portions of these proteins contain a helicase activity that is believed to unwind the cleaved strand (37, 65), while the IncP, IncI, and IncQ relaxases are thought to lack this helicase activity. Unwinding of plasmid DNA in the latter plasmids is believed to be carried out by a host-encoded helicase (32, 67).

The conjugal transfer systems of two IncN plasmids, pKM101 (51, 52, 70–73) and pCU1 (26, 48, 49, 56), have also been described. The pilus-encoding region of pKM101 was previously described at the sequence level and shown to contain 10 genes that are required both for conjugative transfer of pKM101 and for sensitivity to several donor-specific bacteriophages that bind to the plasmid's conjugal pilus (8, 51). Earlier complementation experiments indicated that the remaining portion of the *tra* region contained four complementation groups that are required for conjugation but not required for sensitivity to these phages (70). These complementation groups were therefore thought to direct the processing of plasmid DNA during conjugation. These genes are flanked by the *oriT* and the *fip* gene. *fip* is not required for conjugation but inhibits the fertility of coresident IncP plasmids (72).

Plasmid pCU1 shows striking conservation of restriction sites present in pKM101 over the entire *tra* region, and the nucleotide sequence of the *oriT* region is identical to that of pKM101 (9, 48). These *nic* sites also resemble that of the IncW plasmid R388 (49). The transfer systems of pCU1 and pKM101 are quite similar to that of the IncW plasmid R388 in other

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TABLE 1. Strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or bacteriophage	Description	Reference or source
Strains		
AB1157	F$^-$ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	68
JC2926	AB1157 *recA13*	68
JC7623	AB1157 *recB21 recC22 sbcB15*	68
GW4212	JC7623 *recA*::Cm^r	74
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	57
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 met-1 supE44* λ$^-$	57
POII1734	F$^-$ MudII1734 *lac*$^+$ *ara*::(Mu cts) *araD139* Δ(*lac*)*X74 galU galK rpsL*	5
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE* (Am)*recA1*	38
S17-1	*thi pro hsdR hsdM*$^+$ *recA* RP4 *tra*	59
BL21(DE3)	F$^-$ *ompT* r_B^- m_B^- λDE3	61
PN1	Tn*phoA* in chromosome of CC118	Iyer lab collection
Plasmids		
pTZ18R	*rep*-ColE1, *lacZ*α Ap^r	U.S. Biochemical
pET23d	*rep*-ColE1, T7 promoter, Ap^r	Novagen
pGW276	pKM101 Ω440::Tn*5* deleted across *Sal*I sites	30
pGW277	pKM101 Ω750::Tn*5* deleted across *Sal*I sites	30
pGW2132	*Bgl*II fragment containing *traJ-orfD* cloned into *Bam*HI site of pACYC184	70
pNH-*kan*	*oriT*-RK2 cloned into pUC1318, Km^r Ap^r	22
oriT		
pCU57D14	*traI*, *traJ*, and *traK* genes of pCU1 cloned into pACYC184, Cm^r	48
pMK2004	*rep*-ColE1, Ap^r Tc^r	
pSP34	*traI*, *traJ*, and *traK* genes of pCU1 and *oriT* of RK2 cloned into pACYC184, Cm^r	This study
pSP27	*oriT* of pCU1 cloned into pMK2004, Ap^r Tc^r	This study
pET-*traK*	*traK* of pCU1 cloned into pET23d, Ap^r	This study
pET-*traJ*	*traJ* of pCU1 cloned into pET23d, Ap^r	This study
pET-*traI*	*traI* of pCU1 cloned into pET23d, Ap^r	This study
pSW345	*fip*, *traKJI*, *stbABC*, and *orfD* cloned into pUC12Cm	This study
pMIM101	*traI* and *fip* of pKM101 cloned into pTZ18R, Ap^r	This study
Bacteriophages		
λ::Tn*phoA*	*c*I1857 *b*221 *P*am3 *rex*::Tn*phoA*, Km^r	69
MudII1734	*lacZYA neo c*ts, Km^r	7

respects, including heterologous complementation of *tra* functions and the pattern of bacteriophage sensitivities imparted by their pili (4, 33, 35).

The experiments presented below characterize the conjugal DNA-processing regions of the IncN plasmids pCU1 and pKM101, showing them to be essentially identical at the DNA sequence level. Although this region was previously thought to contain four *tra* complementation groups, only three *tra* genes were identified in the sequence presented in this study. The three genes are transcribed on the same strand, probably from a promoter that lies near or within the *oriT*. The products of these *tra* genes rather strongly resemble their counterparts in the IncW plasmid R388 and more weakly resemble Tra proteins of other plasmids. Directly downstream from these genes is the *fip* gene, whose product is sufficient for the fertility inhibition of coresident IncP plasmids and which may be expressed as part of this putative *tra* operon. We also present the sequence and functional analysis of the leading region of these plasmids, which is located on the opposite side of *oriT*. This region contains three genes whose products appear to be required to prevent plasmid instability that can arise as a consequence of interplasmidic homologous recombination.

MATERIALS AND METHODS

Construction of recombinant plasmids. The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. Standard cloning techniques were employed (57), using buffers and reaction conditions recommended by the enzyme suppliers. pSP34 was constructed by cloning the RK2 *oriT* as a *Bam*HI fragment from pNH-Kan/oriT (22) into the *Bam*HI site of pCU1 derivative pCU57Δ14 (48). pET-*traK* was constructed by cloning the *traK* coding region as a *Bst*YI-*Hpa*I fragment from pSP34 into the *Bam*HI-*Hin*cII sites of pET23d. pET-*traJ* was created by using PCR amplification to create a DNA fragment containing *traJ* flanked by *Bam*HI and *Eco*RI sites and cloning this fragment into the *Bam*HI-*Eco*RI gap of pET23d. pET-*traI* was constructed by cloning the *Bgl*II-*Hin*dIII fragment from pSP34 into the *Bam*HI-*Hin*dIII sites of pET23d. Each of the resulting junctions between the T7 promoter and the *tra* gene was checked by DNA sequencing. Plasmid pMIM101 was created by ligating a 3.5-kb *Bgl*II fragment containing the *traI* and *fip* genes of pKM101 into the *Bam*HI site of pTZ18R such that these genes are transcribed from the P*lac* promoter of the vector. pSW345 was created by digesting pKM101 Ω155::Tn*5* (30) with *Sma*I and *Hin*dIII and inserting the fragment that contains *fip*, *traKJI*, *stbABC*, and *orfD* into the *Sma*I-*Hin*dIII gap of plasmid pUC12Cm.

Transposon mutagenesis. pSP34 was mutagenized with transposon Tn*phoA* (38) by incubating 1 ml of log-phase CC118(pSP34) with 1 ml of a λ::Tn*phoA* lysate (ca. 10^9 phage) overnight at 30°C with gentle shaking. The culture was centrifuged and resuspended in 1 ml of Luria-Bertani (LB) medium, and 0.2 ml of this suspension was spread onto LB plates containing kanamycin (50 μg/ml) and chloramphenicol (60 μg/ml). A 0.5-ml portion of a kanamycin solution (10 mg/ml) was added to the center of each plate and allowed to be absorbed into the agar. The plates were incubated overnight at 37°C. The high level of kanamycin caused a zone of clearing containing a small number of isolated colonies. These colonies were selected for further analysis and found to contain Tn*phoA* derivatives of pSP34. pET-*traI* was mutagenized with Tn*phoA* by transforming it into *Escherichia coli* PN1, which carries Tn*phoA* on the chromosome of strain CC118. The transformation mixture was incubated in LB medium overnight at 37°C and then plated on LB plates containing chloramphenicol and kanamycin. After overnight incubation at 37°C, the confluent colonies were recovered from the plate and plasmid DNA was extracted. A portion of this DNA was introduced into DH5α by electroporation and plated on LB plates containing chloramphenicol and kanamycin. Transformants were found by restriction digestion to contain Tn*phoA* derivatives of pET-*traI*. Plasmids pMIM101 and pSW345 were mutagenized with transposon MudII1734 by published procedures (7).

DNA sequencing. pKM101-derived DNA was sequenced by using derivatives of pMIM101 and pSW345 containing insertions of MudII1734 as template DNA.

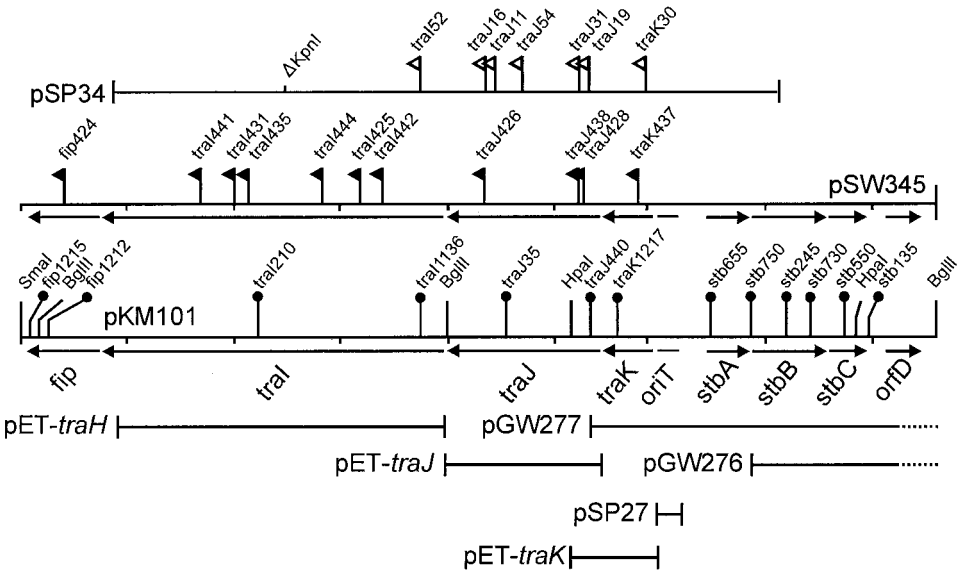

FIG. 1. Genetic map of the conjugal DNA-processing regions of pKM101 and pCU1 and the *stb* region of pKM101. Open triangles, positions and orientations of Tn*phoA* derivatives of pSP34; filled triangles, insertions of MudII1734 in plasmid pSW345; circles, Tn*5* insertion derivatives of pKM101 (70). pGW277 and pGW276 are deletion derivatives of pKM101 (30); solid lines show DNA retained in these plasmids. pSP27 is a derivative of pMK2004 containing *oriT* of pCU1. Plasmids pET-*traK*, pET-*traJ*, and pET-*traI* are derivatives of pET23d that overexpress the corresponding *tra* products. Short vertical lines indicate the scale, measured in kilobases.

These plasmids were purified by using SpinBind columns (FMC Bioproducts) and sequenced by using a 373A Stretch DNA sequencer (ABI) and primers that hybridize to the left or right end of MudII1734. Sequencing reactions were carried out on both DNA strands with *Taq* DNA polymerase and DyeDeoxy Terminator Sequencing kits (ABI) and deoxynucleoside triphosphate substrates. Custom-made primers that hybridize to *traI* or *fip* DNA were used as needed to complete the sequence.

pCU1-derived DNA was sequenced either manually, using a modification of the U.S. Biochemical Sequenase protocol described previously (49), or by automated DNA sequencing with a 373A Stretch DNA sequencer (ABI). Sequences was determined from both strands by sequencing outward from pSP34::Tn*phoA* or pET-*traI*::Tn*phoA* inserts with primers that hybridize to *phoA* or to IS*50* DNA. In the latter case, a restriction fragment containing DNA from only the right IS*50*, and hence only one primer site, was extracted from an agarose gel via use of GeneClean (Bio/Can Scientific). *traI* was sequenced by using plasmid pSP60 as a template and custom-made oligonucleotide primers. Inferred protein sequences were used to search public protein sequence databases by using the BLAST algorithm (1).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Derivatives of *E. coli* BL21(DE3) containing a derivative of pET23d containing individual *tra* genes were cultured to an optical density at 590 nm of 0.4 in M9 medium supplemented with ampicillin (30 μg/ml), thiamine (40 μg/ml), and all 20 amino acids (40 μg/ml) except methionine and cysteine. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to some cultures to a final concentration of 0.4 mM, and incubation was continued at 37°C for 40 min, at which time rifampin (200-μg/ml final concentration) was added. After a further 20 min at 37°C, 10 μCi of [^{35}S]methionine was added to the culture, and incubation was continued at 37°C for 20 min. One milliliter of each culture was centrifuged, resuspended in 50 μl of protein loading buffer, and boiled for 5 min. The lysate was centrifuged for 5 min in a microcentrifuge, and 5 μl of the supernatant was loaded onto a sodium dodecyl sulfate (SDS)–12.5% discontinuous polyacrylamide gel (20). The gel was stained with Coomassie blue and autoradiographed with BioMax X-ray films (Dupont).

Genetic complementation of *tra* mutations. Derivatives of strain JC2926 containing a derivative of pKM101 and a derivative of pSW345 were cultured in LB medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml) to an optical density at 600 nm of approximately 0.5. A single culture of the conjugal recipient (MM294) was cultured to a similar optical density and concentrated 50-fold by centrifugation. Fifty microliters of each donor culture was combined with 50 μl of the concentrated suspension of MM294 and spotted

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pET23d                                          BamHI EcoRI      HincII  HindIII
AAGGAGATATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCCGAATTCGAGCTCCGTCGACAAGCTTG...
 RBS          M  A  S  M  T  G  G  Q  Q  M  G  R  I  R  I  R  A  P  S  T  S  L  ...

pET-traK                                    BamHI/BstYI                          RBS
AAGGAGATATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGgatccaatcttttgtttaagaaatgcacaatgtgaggactgaatgccaataataa..
 RBS          M  A  S  M  T  G  G  Q  Q  M  G  R  I  P  I  F  C  L  R  N  A  Q  C  E  N  *
                                                                                  M  P  I  I ...

pET23-traJ                                      BamHI       RBS
AAGGAGATATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATccaaaaaaggaggcgctgaaatggacgatagagaaaga
 RBS          M  A  S  M  T  G  G  Q  Q  M  G  R  I  Q  K  R  S  A  E  M  D  D  R  E  R

pET-traI                                    BamHI/BglII
AAGGAGATATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGgatctgatgcttcatataa...
 RBS          M  A  S  M  T  G  G  Q  Q  M  G  R  I  *
                                                  M  L  D  I  T  T  ...
```

FIG. 2. 5′ ends of pCU1 *tra* genes cloned into the pET23d expression vector. Nucleotide sequences are shown in the upper lines, with corresponding amino acid sequences below. pCU1 nucleotide sequences are shown in lowercase letters, while pET nucleotide sequences are shown in uppercase letters. The amino acid sequences derived from pET23d are shown in roman type, while the amino acid sequences derived from pCU1 genes are italic. Restriction sites, ribosome binding sites (RBS), and putative ATG start codons are underlined.

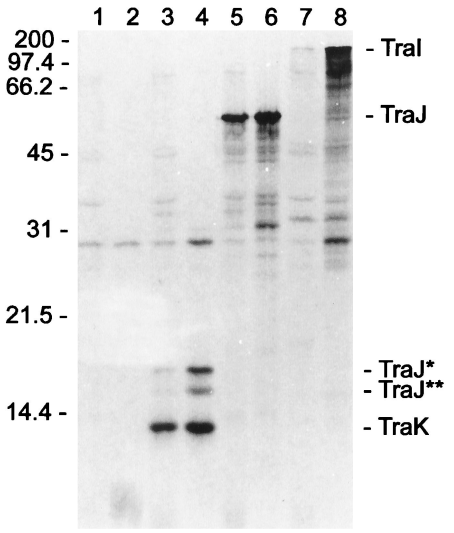

FIG. 3. SDS-PAGE of pCU1 Tra proteins. Positions of molecular mass standards (in kilodaltons) are indicated at the left. Lanes 1 and 2, pET23d; lanes 3 and 4, pET-*traK*; lanes 5 and 6, pET-*traJ*; lanes 7 and 8, pET-*traI*. The cultures used to make the cell extracts in lanes 2, 4, 6, and 8 were treated with 0.4 mM IPTG prior to addition of rifampin. Proteins corresponding to predicted Tra proteins are indicated at the right.

onto Millipore filters that had been placed on prewarmed LB agar medium. These plates were returned to a 37°C incubator for exactly 1 h, at which time the cells were resuspended in 1% NaCl. These cells were serially diluted and plated onto defined medium (AB salts and buffer) containing chloramphenicol (50 μg/ml) to select for transfer of pSW345 derivatives into MM294. Donor cells were enumerated by plating on LB agar supplemented with chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), and streptomycin (250 μg/ml). Transfer efficiency was calculated as the number of recovered transconjugants per recovered donor per hour.

Plasmid cointegration assays. Filter matings between recipient strain HB101rif and donor strain S17-1 containing pSP27 and a derivative of pSP34 were conducted as previously described (48). pSP34 contains the *oriT* site of plasmid RK2 and is therefore efficiently mobilized by the *tra* system of S17-1. pSM34 and pSP27 also contain the *oriT* of pCU1, and the assay measures the efficiency of cointegration between these sites. Transconjugants were selected on LB plates containing rifampcin (100 μg/ml) and either chloramphenicol (60 μg/ml) to select for transfer of pSP34 or tetracycline (20 μg/ml) to select for cointegrative transfer of pSP34 and pSP27.

Plasmid curing assays. Strains were cultured from frozen permanent stocks into LB medium containing ampicillin (50 μg/ml) to minimize the accumulation to plasmid-free cells. When these cultures had reached saturation, they were serially diluted 10^4-fold, and 0.1 ml of the resulting cell suspension was used to inoculate a 10-ml culture of LB medium. Cultures were incubated to the stationary phase (approximately 20 generations) in 125-ml Erlenmeyer flasks with vigorous aeration. They were then serially diluted 10^6-fold in 100-fold increments. A 0.1-ml portion of the 10^4-fold dilution was used to inoculate a fresh 10-ml LB culture, while 0.1 ml of the 10^6 dilution was plated to determine the fraction of plasmid-free colony-forming units. The procedure was repeated two additional times (60 generations total).

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the GenBank DNA sequence database (accession no. U43676, AF000361, and AF109305).

RESULTS

The conjugal DNA-processing regions of pKM101 and pCU1. The DNA sequence of an 8.7-kb region between the *Sma*I-1 site and *Bgl*II-3 site of plasmid pKM101 (30) revealed eight open reading frames (ORFs) that are likely to encode proteins (Fig. 1). Four of these ORFs (designated *traK*, *traJ*, *traI*, and *fip*) are transcribed from right to left, while the remaining four ORFs (*stbA*, *stbB*, *stbC*, and *orfD*) are transcribed from left to right. *traK* and *stbA* are separated by a 513-nucleotide intergenic region that contains the *oriT*. The intergenic regions between the *traK*, *traJ*, *traI*, and *fip* genes contain 1, 10, and −1 nucleotides, respectively, suggesting that these genes are probably transcribed as an operon from a promoter that lies within or near *oriT*. The region containing *traK* to *fip* was sequenced independently in the laboratory of one of the authors (R.W.), who obtained an identical sequence (GenBank AF0000361). We also sequenced the *traK*, *traJ*, and *traI* genes of pCU1 (3.4 kb in all), and found that these sequences were identical to those of pKM101 at all but three bases in *traI*. One of the DNA sequence differences altered TraI residue 206 from alanine to threonine. The two remaining DNA sequence differences did not alter the protein sequence.

Visualization of the TraK, TraJ, and TraI proteins. The *traK*, *traJ*, and *traI* genes of pCU1 were each cloned individually into the expression vector pET23d (Fig. 2). These constructs were introduced into *E. coli* BL21(DE3) to visualize the corresponding Tra proteins by SDS-PAGE. Plasmid pET-*traK* contains 43 bp upstream of the putative ATG start codon (Fig. 2) and hence should produce a native TraK protein (with 139 amino acids and a molecular mass of 15.3 kDa). pET-*traK* also contains 113 codons of *traJ* translationally fused to 33 codons of the pET23d vector (146 codons in all, encoding a peptide of 16.6 kDa). This plasmid directed the synthesis of three detectable proteins (Fig. 3, lane 4). The two most strongly expressed proteins (14.3 and 18 kDa) probably correspond to the native TraK protein and the TraJ fragment, respectively. The fainter protein (16 kDa) could be a truncated version of the TraJ fragment initiated from an internal start codon 20 codons downstream of the ATG used to initiate the 18-kDa TraJ peptide (Fig. 2). The detection of the TraJ peptides strongly suggests that *traK* and *traJ* are translationally coupled and therefore are expressed from a single promoter.

pET-*traJ* contains the native *traJ* coding region and ribosome binding site. Directly upstream of the start codon are 19 additional codons, most of which are derived from the plasmid vector (Fig. 2). The 58-kDa protein observed on SDS-PAGE

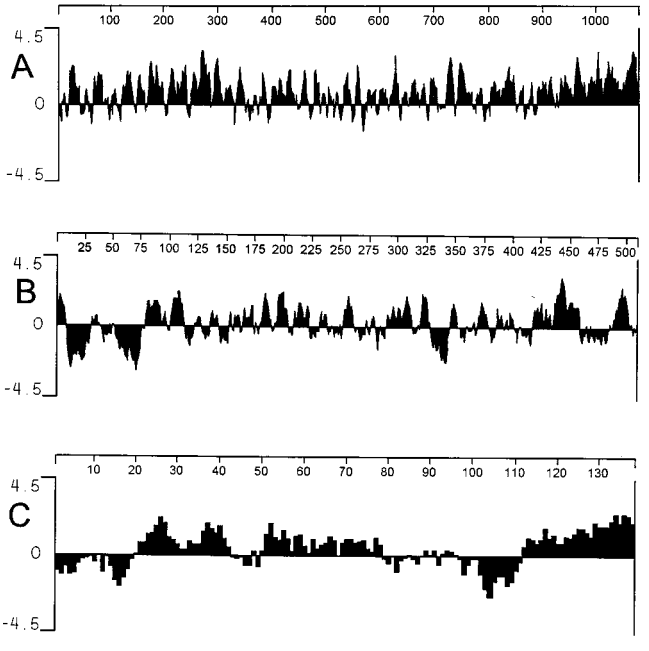

FIG. 4. Hydropathy profiles of the TraI (A), TraJ (B), and TraK (C) proteins. The algorithm of Kyte and Doolittle (28) was used.

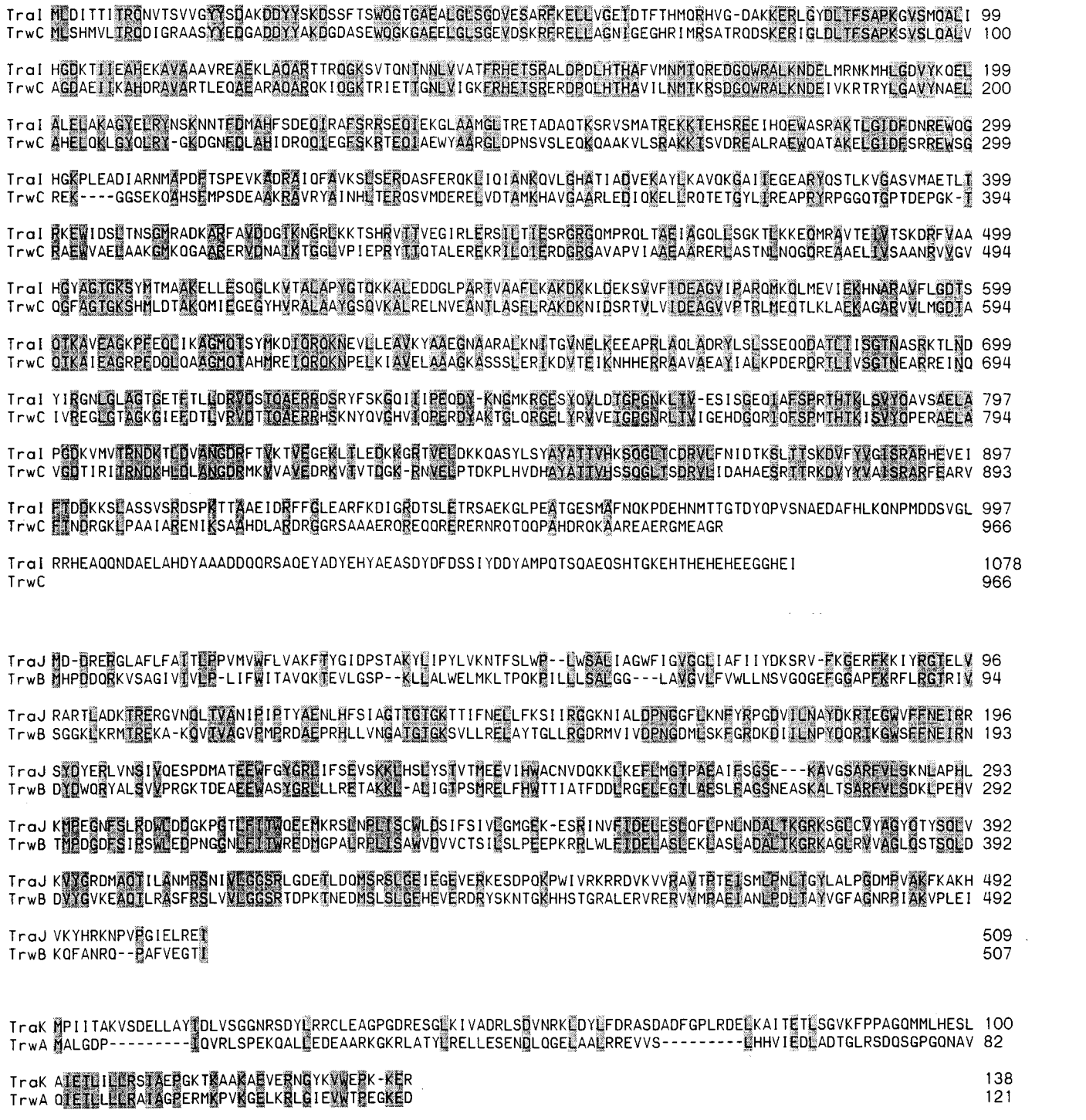

FIG. 5. Protein sequence similarity between the Tra proteins of pKM101 and the Trw proteins of plasmid R388. The Clustal method (23) was used, with a gap penalty of 20 and a gap length penalty of 20.

(Fig. 3, lane 6) correlates well with the predicted molecular mass (59 kDa) of this TraJ fusion protein.

Plasmid pET-*traI* was used to visualize the 1,078-amino-acid TraI protein. However, the *traI* gene of this plasmid contains a 14-codon truncation at its 3′ end. The remainder of *traI* (codons 1 to 1064) is translationally fused at its 3′ end to 42 codons of an ORF of the pET23d vector, resulting in a fusion protein having a molecular mass of 123 kDa. Although pET23d was designed to provide a ribosome binding site and start codon, this start codon is out of frame with respect to *traI*, and translation of *traI* must therefore originate from the predicted *traI* start codon, which is preceded by a rather weak ribosome binding site (GGA). pET-*traI* expresses a single protein with a molecular mass of 155 kDa (Fig. 3, lane 8), indicating that this

TABLE 2. Complementation of mutations in the conjugal DNA-processing region of pKM101

pSW345 derivative	pKM101 derivative[a]:			
	traI210	*traI1136*	*traJ35*	*traK1217*
traI441	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	ND[b]	ND
traI431	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	ND	ND
traI435	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	2.7×10^{-1}	8.9×10^{-1}
traI444	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	5.0×10^{-1}	ND
traI425	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	3.3×10^{-1}	ND
traI442	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	5.6×10^{-1}	5.3×10^{-1}
traJ426	3.8×10^{-1}	3.8×10^{-1}	$<1.0 \times 10^{-5}$	5.0×10^{-1}
traJ438	ND	5.7×10^{-1}	$<1.0 \times 10^{-5}$	6.7×10^{-1}
traJ428	ND	6.8×10^{-1}	$<1.0 \times 10^{-5}$	3.3×10^{-1}
traK437	3.9×10^{-1}	6.9×10^{-1}	9.1×10^{-1}	$<1.0 \times 10^{-5}$
fip424	1.2	1.8	1.6	1.3

[a] Values represent numbers of transconjugants per donor per hour.
[b] ND, not determined.

DNA fragment encodes one large protein. This finding confirms the nucleotide sequence analysis but is difficult to reconcile with earlier complementation analysis (70), which predicted that this region contains two complementation groups rather than one (see Discussion).

Amino acid sequence analysis of pCU1 and pKM101 DNA-processing proteins. The *traK*, *traJ*, and *traI* genes encode proteins that are predicted to be largely hydrophilic, suggesting that they are soluble in aqueous environments. However, TraJ contains a strongly hydrophobic region between residues 10 and 25 (Fig. 4), which is preceded by arginine residues at residues 4 and 6. This suggests that the amino terminus of TraJ may be exported from the cytoplasm by the general protein export system (41). TraJ contains a second hydrophobic region between residues 54 and 74 followed by positively charged residues at positions 77, 79, 82, 85, 87, 88, and 91. This sequence resembles a stop transfer signal, suggesting that TraJ may have a transmembrane topology, with residues 26 to 53 located in the periplasmic space and the remainder of the protein located in the cytoplasm.

We used the TBLASTN algorithm (1) and the GenBank DNA sequence database to identify proteins having protein sequence similarity to TraK, TraJ, and TraI, and we found extensive sequence similarity to the products of a variety of conjugal transfer genes. The most closely related proteins are TrwA, TrwB, and TrwC of the IncW plasmid R388 (35). TraK and TrwA are 22% identical, with virtually all similarity limited to the carboxyl termini of the proteins (Fig. 5). Similarly, TraJ and TrwB are 37% identical, with similarity distributed along the entire lengths of these proteins. Like TraJ, TrwB is predicted to have a transmembrane topology (35). Finally, TraI and TrwC are 43% identical over their entire lengths, although similarity seems to be strongest at the proteins' amino termini (Fig. 5). TraI is 108 residues longer than TrwC at its carboxyl terminus, and this nonconserved region contains many acidic amino acid residues. A-Tn*9* insertion mutation in this region reduced but did not abolish conjugation (70). TrwA, TrwB, and TrwC are involved in processing of plasmid DNA during conjugation (35). They have been the subject of extensive sequence, genetic, and biochemical analysis, which is summarized in Discussion.

Complementation analysis. Previous complementation studies with pKM101::Tn*5* insertion mutants identified five complementation groups: *traK*, *traJ*, *traH*, *traI*, and *fip*. Mutations in the region from *traH* to *traK* eliminated transfer, while mutations in *fip* abolished fertility inhibition of the IncP plasmids (70, 72). None of these genes was required for phage sensitivity, suggesting that none is required for synthesis of the conjugal pilus. In those earlier studies, complementation analysis was carried out by using transient heterozygotes that contained two Tn*5* derivatives of pKM101, one introduced by transformation. Since the DNA sequence of this region indicates that there are three genes rather than four, we repeated this analysis with stable merodiploids. To do this, a region of pKM101 DNA (Fig. 1) was subcloned into pUC12Cm, creating pSW345. This plasmid was subjected to transposon mutagenesis with MudII1734 (7), and 10 derivatives having insertions in *tra* genes were isolated. These were introduced into derivatives of *E. coli* MC4100 containing *tra* mutants of pKM101, and the resulting merodiploids were tested for the ability to transfer kanamycin resistance to a conjugal recipient. These experiments indicate that this region contains three complementation groups rather than four (Table 2). The mutations previ-

TABLE 3. Mobilization of pSP27 by pSP34 *tra* mutants from S17-1

Mobilizing plasmid	Mobilized plasmid	Mobilization frequency[a]	No. of transconjugants with the following plasmid structure:			
			Cointegration at *oriT*	Cointegration in vector	Parental	Other
pSP34	pMK2004	9.8×10^{-6}	0	5	7	0
	pSP27	2.4×10^{-3}	21	0	4	0
pSP34 *traI52*	pSP27	4.6×10^{-6}	3	7	6	0
pSP34 *traI*ΔKpn	pSP27	2.4×10^{-7}	2	0	2	1
pSP34 *traJ16*	pSP27	4.7×10^{-4}	1	0	9	0
pSP34 *traJ11*	pSP27	5.4×10^{-5}	6	0	2	0
pSP34 *traJ54*	pSP27	5.1×10^{-4}	3	0	2	0
pSP34 *traJ31*	pSP27	2.9×10^{-4}	5	0	0	0
pSP34 *traJ19*	pSP27	1.7×10^{-4}	7	0	2	1
pSP34 *traK30*	pSP27	1.0×10^{-5}	3	4	4	3

[a] Mobilization frequencies are given as the number of transconjugants receiving pSP27 (or pMK2004) per transconjugant receiving the pSP34 derivative. In this assay, pSP34 (and derivatives) is efficiently transferred via its RK2 *oriT* sites and the RK2 *tra* genes on the chromosome. Transfer of pSP27 requires cointegration with pSP34 via the pCU1 *oriT* sites found on both plasmids.

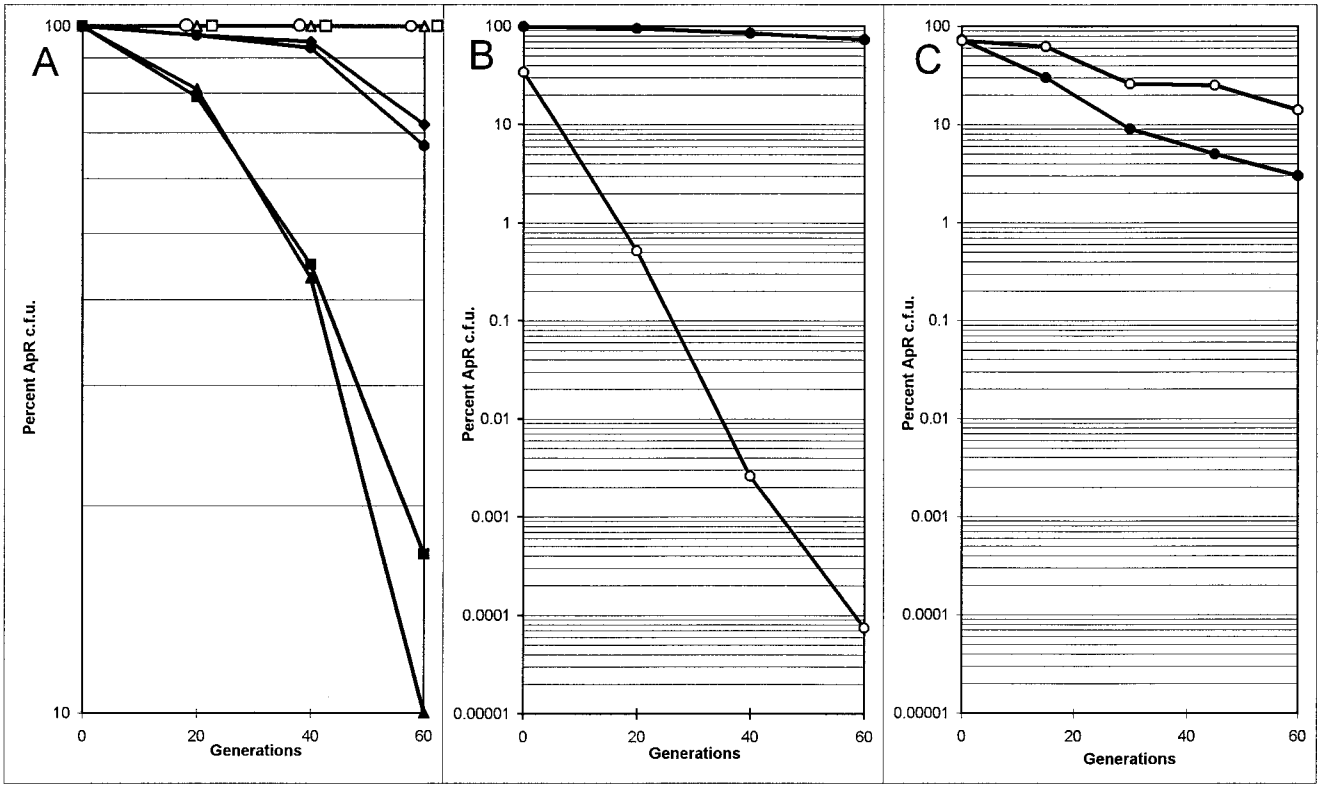

FIG. 6. Curing of pKM101 and its derivatives during prolonged culturing in the absence of antibiotic selection. (A) AB1157(pKM101) (●), AB1157(pKM101 *stbA655*::Tn*5*) (■), AB1157(pKM101 *stbC135*::Tn*5*) (▲), AB1157(pKM101 Ω155::Tn*5*) (◆), JC2926(pKM101) (○), JC2926(pKM101 *stbA655*::Tn*5*) (□), and JC2926(pKM101 *stbC135*::Tn*5*) (△). (B) JC7623(pGW277) (●) and JC7623(pGW276) (○). (C) GW4212(pGW277) (●) and GW4212(pGW276) (○).

ously designated *traH* alleles have therefore been renamed *traI* alleles.

Complementation experiments between Tn*5* mutants of pSP34 and Tn*5* mutants of pKM101 were also conducted (data not shown). As expected, pSP34 was able to complement each of the pKM101 Tn*5* mutants. Complementation experiments between pKM101::Tn*5* derivatives and pSP34::Tn*5* derivatives produced results similar to those obtained with pKM101::Tn*5* and pSW345::MudII1734 derivatives (data not shown).

Intracellular site-specific recombination at *oriT*. Intramolecular site-specific recombination between two *oriT*s on the same plasmid has been observed previously and used as an assay for *oriT*-processing activity (5, 6). In this study, an assay involving intermolecular site-specific recombination between *oriT*s carried on two separate plasmids was used to determine which of the *tra* functions are required for cleavage and religation at *oriT* during transfer.

In this assay, Tn*phoA* mutants of pSP34 were tested for their ability to mobilize a second plasmid (pSP27) containing the *oriT* of pCU1 from the *E. coli* donor strain S17-1 into the *E. coli* recipient strain HB101rif. pSP34 carries the *traK*, *traJ*, and *traI* genes and *oriT* of pCU1. This construct also carries the *oriT* region of IncP plasmid RK2, which allows it to be mobilized efficiently by the IncP *tra* system carried on the chromosome of strain S17-1. Mobilization of pSP27 from S17-1(pSP34)(pSP27) occurs via a process known as conduction (54), in which pSP27 is integrated into pSP34 at the IncN *oriT* sites of both plasmids. The resulting cointegrate plasmid is then mobilized by transfer initiated at the IncP *oriT*. This process requires (i) some or all of the pCU1 *tra* genes on pSP34 (see below); (ii) the RK2 *oriT* of pSP34, since pCU57D14, which lacks this site, is not mobilized efficiently from S17-1 (data not shown); and (iii) the pCU1 *oriT* of pSP27, since pMK2004 lacks this site and is not efficiently mobilized (Table 3).

The mobilization frequency of pSP27 was decreased to various extents by *tra* mutations in pSP34 (Table 3). Insertions in *traI* reduced mobilization of pSP27 by 1,000-fold, while the *traK* insertion caused a 100-fold reduction and *traJ* mutations caused only a 10-fold reduction. It seemed possible that not all transconjugants would have plasmids cointegrated at *oriT*. To test this, we took advantage of the fact that the *oriT* of pSP34 is contained on a 2.3-kb *Hpa*I fragment, while pSP27 (5.4 kb in length) has no *Hpa*I sites. Cointegration at *oriT* would therefore create a plasmid containing a diagnostic 7.7-kb *Hpa*I fragment. When S17-1(pSP34)(pSP27) was used as a donor, 84% of the transconjugants (21 of 25) contained a plasmid with a 7.7-kb *Hpa*I fragment. By comparison, when S17-1(pSP34)(pMK2004) was used as a donor, none of the transconjugants had such a fragment, indicating that mobilization of pMK2004 must have occurred by some other mechanism. Integration into the vector portion of the plasmid could also occur via recombination at the *oriV* sites on either plasmid, as described by Reimmann and Haas (54). The presence of two unaltered parental plasmids would presumably result from the resolution of such a cointegrate following transfer.

As described above, mutations in the *tra* genes of pSP34 decreased but did not abolish the mobilization of pSP27. However, most of the transconjugants did not have plasmids that had cointegrated via *oriT* sites. When the donor contained a *traK* mutation, only 19% of the transconjugants (3 of 16) had plasmids cointegrated at *oriT*. Similarly, when the donor con-

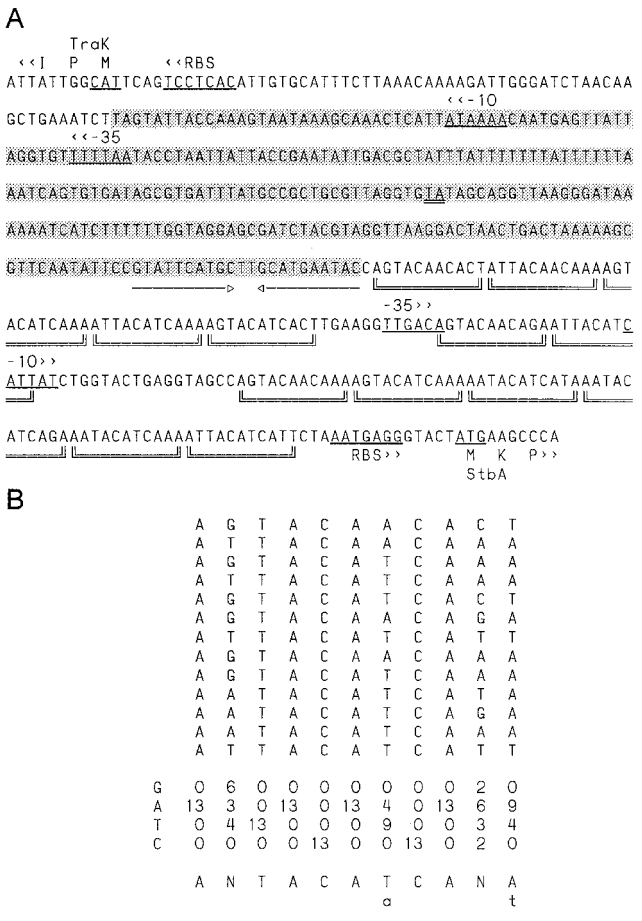

FIG. 7. DNA sequence of the intergenic region between *traK* and *stbA*. (A) Putative promoters, ribosome binding sites (RBS), and translation start sites for *traK* and *stbA* are underlined. The *oriT* sequence is shaded, while the nucleotides that flank the proposed *nic* site are double underlined. A dyad symmetry is indicated by inverted arrows, and repeated sequences are indicated by square brackets. (B) Alignment of the repeated sequences.

tained a *traI* mutation, only 24% of the transconjugants (5 of 21) had this structure. In contrast, when the donor strain contained a *traJ* mutation, 58% of the transconjugants (22 of 38) had plasmids cointegrated at their *oriT* sequences. These results provide further support for the idea that both *traK* and *traI* are important for cointegration and hence for cleavage and resealing of *oriT* sites during conjugation, while *traJ* does not play a central role.

A locus required for stable plasmid inheritance. Transposon insertions within a 1.5-kb region of pKM101 were found somewhat serendipitously to decrease the stable inheritance of the plasmid, causing plasmid-free bacteria to accumulate during prolonged culturing. This locus was first described as being required for sensitivity to the donor-specific bacteriophages Ike and PRD1, even though it is not required for efficient conjugation (unpublished data). It was subsequently found that strains containing *stb* mutants of pKM101 formed wild-type plaques on media containing kanamycin or ampicillin, while plaques were not detectable on antibiotic-free media. This was thought to be due to plasmid-free, phage-resistant bacteria overgrowing and obscuring the plaques. Additional alleles of this locus were later identified during two subsequent screens, one for deficiency in the fertility of coresident IncP plasmids (the Fip phenotype) and another for deficiency in entry exclusion (the Eex phenotype). However, when plasmid-free bacteria were eliminated (either by using antibiotics or, as described below, by altering the host genotype), mutants with transposon insertion mutations in this region were found to be Fip^+ and Eex^+.

We quantitated the rate of loss of Ap^r from cells containing pKM101 and its *stb* derivatives. Two strains containing *stb*::Tn*5* derivatives of pKM101 lost Ap^r at a 10-fold-higher rate than pKM101 itself (Fig. 6A). Both curves tend to arc downwards, suggesting that plasmid-free cells may also have a slightly higher division rate than plasmid-containing cells. These data indicate that about 4% of the cells of a culture containing an *stb* mutant plasmid became Ap^s per generation, while cells containing pKM101 became Ap^s at a rate of about 0.3% per generation.

We attempted to complement this deficiency by using pGW2132 (a derivative of pACYC184 containing the *traJ*, *traK*, *stbABC*, and *orfD* genes of pKM101, [70]). The complementation assays described above were originally carried out within a *recA* strain of *E. coli*. To our surprise, *stb* pKM101 derivatives were fully stable in this genetic background, even in the absence of a second plasmid (see below). These assays were therefore repeated with a recombination-proficient host, and it was found that pGW2132 did not increase the stability of coresident *stb* derivatives of pKM101 (data not shown). However, interpretation of this result is difficult because both pGW2132 and its parent pACYC184 appeared to destabilize pKM101. Therefore, nonspecific competition between these two plasmids or other interactions may have obscured any complementation.

We subsequently compared plasmid stability in a $RecA^+$ host (AB1157) and an isogenic $RecA^-$ host (JC2926). In the *recA* strain, we did not detect loss of either pKM101 or an *stb* insertion derivative even after 60 generations (Fig. 6A, open symbols). We conclude that plasmid instability requires homologous recombination and that proteins encoded by the *stb* locus prevent this recombination-dependent instability.

Several multicopy plasmids have been observed to be particularly unstable in a *recB recC sbcB* strain (27). The stabilities of several pKM101 derivatives were therefore tested in this background. These tests were done with a set of deletion derivatives of pKM101 rather than transposon insertion derivatives. When we attempted to introduce these plasmids into strain JC7623 by transformation, we found that pKM101 itself and deletion derivatives that retained *stb* could be readily introduced but that all deletion derivatives lacking *stb* yielded either no transformants or a small number of slow-growing colonies (data not shown). We quantitated the stabilities of pGW276 (*stb*) and pGW277 (stb^+) (Fig. 1). The former plasmid was lost at a rate of 20% per generation, while the latter plasmid was lost at a rate of only 0.5% per generation (Fig. 6B). To determine whether this instability was dependent upon homologous recombination, we introduced the same two plasmids into GW4212, which contains mutations in the *recB*, *recC*, *sbcB*, and *recA* genes (69). Although both strains lost Ap^r at detectable rates, the two plasmids seem to be about equally stable (Fig. 6C). If anything, pGW276 (which lacks *stb*) appeared to be slightly more stable in this host than pGW277, although this difference was very slight. We conclude that the plasmid instability observed in JC7623(pGW276) is dependent upon a recombination-proficient genotype.

Sequence analysis of this region revealed four ORFs, designated *stbA*, *stbB*, *stbC*, and *orfD*, encoding products having predicted molecular masses of 15.7, 26.4, 13.5, and 13.2 kDa, respectively. These ORFs are identical to the *orfA*, *orfB*, *orfC*,

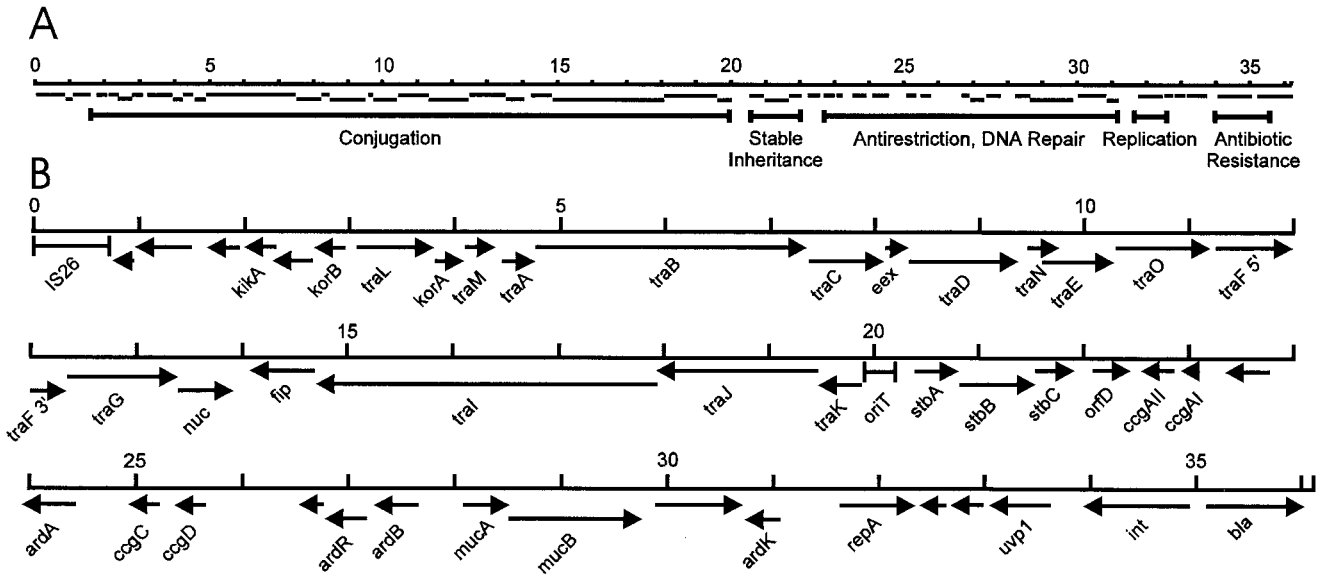

FIG. 8. Functional map (A) and physical map (B) of pKM101. Labelled arrows denote characterized genes, while unlabelled arrows denote uncharacterized ORFs. Previous maps of pKM101 started at the unique *Eco*RI site (31). To avoid confusion between the locations of genes in pKM101 and its parental plasmid R46, we have numbered the sequence from the first nucleotide of IS*26* (which is common to both plasmids). Gene designations: *bla*, oxa2 β-lactamase (19); IS*26*, insertion sequence IS*26*, (formerly denoted IS*46*); *kikA*, required for killing of *Klebsiella* strains during conjugation (24); *traM* to *-G*, required for conjugation and for sensitivity to donor-specific bacteriophages (51); *traI* to *-K*, conjugal DNA processing (this study); *korA* and *korB*, corepressors of the *korB*, *traL*, and *traN promoters* (40)*; eex*, entry exclusion (52); *nuc*, periplasmic endonucleolytic DNase (53); *fip*, fertility inhibition of coresident IncP plasmids (70); *stbA* to *-C*: stable plasmid inheritance (this study); *ardA* and *ardB*, inhibition of host DNA restriction enzymes (3); *ardR* and *ardK*, regulators of *ardA*, *ardB*, *ccgAI*, *ccgAII*, *ccgC*, *ccgD*, and *repA* (10); *ccgAI*, *ccgAII*, *ccgC*, and *ccgD*, genes of unknown function that are regulated by ArdK and ArdR (10); *mucA* and *mucB*, error-prone DNA repair (50); *mpr*, unknown function, possible metalloprotease (10); *repA*, plasmid vegetative replication (10); *uvp1*, site-specific recombinase (64); *int*, conserved gene within integron (19). The complete DNA sequence of pKM101 was compiled by using sequence data contained in the GenBank DNA sequence database (accession no. AF000360, U09868, U43676, U72482, U00430, U00434, L09114, M81860, Y00358, and X06046).

and *orfD* described by Delver and Belogurov (10). Insertions in *stbA*, *stbB*, or *stbC* confer the unstable plasmid segregation phenotype, while an insertion in *orfD* did not affect stability (data not shown). The *stbA* and *stbB* genes overlap by 17 nucleotides, and *stbB* and *stbC* are separated by 1 nucleotide, suggesting that these three genes are transcribed as an operon from a promoter that lies near *oriT*. In contrast, *orfD* is separated from *stbC* by 181 nucleotides, suggesting that expression of this gene would require a promoter located within this intergenic region. All four proteins contain predominantly hydrophilic amino acid residues, although the carboxyl terminus of StbC is hydrophobic. None of the products of these four ORFs showed any significant sequence similarity to other known proteins.

The DNA sequence just upstream of *stbA* contains a striking pattern of repeated DNA sequences (Fig. 7A). Thirteen such direct repeats were found, each of which showed a remarkable similarity to a consensus sequence (Fig. 7B). Directly upstream of these repeats is an extensive dyad symmetry (inverted arrows in Fig. 7A) and the *oriT* sequence (shaded residues in Fig. 7A). Directly downstream of these repeats is the putative ribosome binding site for *stbA*. Within the region containing these repeats is a possible promoter for the *stb* operon (underlined). Therefore, if these repeats provide a binding site for one or more Stb proteins, binding of these proteins might plausibly repress transcription of the *stbA* promoter.

DISCUSSION

Restriction maps of pCU1 and pKM101 suggested that they contain very similar conjugal transfer regions. The data presented in this paper demonstrate that their conjugal DNA-processing regions are identical at all but 3 nucleotides over a 5,879-bp region encompassing *oriT*, *traK*, *traJ*, and *traI*. pCU1 and pKM101 also have strong sequence similarity at the 2,366-bp *kik* region, which has been sequenced for both plasmids in previous studies (51, 55). The variation observed over both regions represents a substitution rate of less than 0.2%. This high degree of sequence identity at both regions combined with the lack of any restriction site polymorphisms over the entire *tra* region strongly suggests that the *tra* regions of pCU1 and pKM101 are extremely similar and diverged from a common ancestor relatively recently. Furthermore, it is possible that this identity extends past the *tra* region. pCU1 and pKM101 have identical restriction maps except for the regions that encode antibiotic resistances. pKM101 is a deletion derivative of a larger plasmid, R46, which contains resistance determinants against ampicillin, streptomycin-spectinomycin, sulfanomides, tetracycline, and arsenate. Some of these genes are found within an integron (18). In contrast, pCU1 confers resistance to ampicillin and streptomycin-spectinomycin. It is possible that the only significant differences between pCU1, pKM101, and R46 are found in these clustered antibiotic resistance determinants. The deletion that created pKM101 appears to have been mediated by insertion sequence IS*26* (17, 31). It is possible that pCU1 was also derived from an R46-like plasmid by a similar in vivo deletion.

The nucleotide sequences of the conjugal DNA-processing genes of pKM101 and pCU1 identify three genes in this region that are required for conjugation. TraI is homologous to TrwC of the IncW plasmid R388 (Fig. 5), and both proteins are also related to the TraI protein of plasmid F. Both the R388 and F plasmid proteins are known to contain an amino-terminal *oriT*-specific nucleolytic function and a carboxyl-terminal helicase

function (36, 37, 65). The intermolecular-recombination results presented in this study strongly suggest that the IncN TraI has a comparable nucleolytic activity. The corresponding relaxases of the TraI protein of plasmid RK2 and the VirD2 protein of *Agrobacterium tumefaciens* have tyrosine residues (at residues 22 and 29, respectively) which are involved in covalent binding of the protein to the 5′ end of the cleaved strand and are considered to be part of the catalytic centers of these enzymes (46, 47, 66). The IncN TraI protein has four tyrosine residues at positions 18, 19, 26, and 27. This arrangement is reminiscent of the two tyrosine residues separated by three amino acids in the A protein of phage ϕX174, which is required for rolling-circle replication (21). For the ϕX174 system, it was postulated that the two tyrosine residues alternate in cleaving within the replication origin. This situation could assist in the intermolecular recombination event observed in this study. It is conceivable that each tyrosine residue (or one from each pair) cleaves the DNA strand and binds to separate pSP27 and pSP34 *oriT*s, bringing them into close proximity to each other to allow the transesterification step between the free 3′ OH of one plasmid and the 5′ phosphate of the other, resulting in the formation of a cointegrate plasmid.

Intermolecular recombination at the pCU1 *oriT* required both the *traI* and *traK* genes, suggesting that TraK may be a functional homologue of the IncF TraY protein. The TraY proteins of both the F plasmid and R100 are required for DNA cleavage both in vivo and in vitro (25, 42) and for *oriT*-mediated recombination (6). Alignment of the TraK protein with the F TraY protein shows little sequence similarity. On the other hand, there is limited sequence identity, primarily in their carboxyl termini, between TraK and the TrwA protein of IncW plasmid R388. TrwA is not required for intramolecular recombination between two R388 *oriT* sites in vivo (33) but has been shown to enhance in vitro cleavage by TrwC (39). The fact that R388 TrwA is not required for recombination at *oriT* while pKM101 TraK is required may be related to the sensitivities of these two recombination assays. In the IncW study, recombination was identified by the loss of an antibiotic marker situated between two copies of the *oriT* (33). Detection of a recombination event required that all copies of the plasmid within the cell carry the deletion. As a result, individual recombination events within the cell could be masked by the presence of plasmids that had not undergone recombination. The assay described in this study selects for plasmids which have undergone recombination, since only those pSP27 plasmids which cointegrate into the larger pSP34 plasmid are transferred.

TraJ is the only Tra protein in this region whose hydropathy profile suggests a transmembrane topology. Homologous proteins are found in virtually all conjugation systems. Members of this family of proteins have similar hydropathy profiles and in some cases have been shown to be associated with the inner membrane (43). It has been postulated that such a protein could be used to bind both the relaxosome and the mating pore and, by doing so, to bring the DNA in juxtaposition to the pore. This could explain why *traJ* mutations caused such a slight reduction in conduction of pSP27. Binding of pSP27 and pSP34 DNA to the membrane via TraJ prior to cleavage could enhance intermolecular recombination by bringing the two plasmids into closer proximity. Involvement of the *traJ* function in the site-specific recombination event has not been observed for the TraJ homologue of any other transfer system studied to date (33, 42, 46). Alternatively, *traJ*::Tn*5* mutations could exert polar effects on expression of the downstream *traI* gene, although these same mutations did not appear to be polar in complementation assays.

Downstream of *traI* is a gene designated *fip*, which abolishes the conjugation of coresident IncP plasmids (72). *fip* appears to be part of the *traKJI* operon, although the significance of this coexpression is not understood. Immediately downstream from *fip* is the *nuc* gene, which is transcribed convergently to *fip* (53). Like *fip*, *nuc* is also the last gene in a *tra* operon and has no direct role in conjugation.

It was initially surprising that three *tra* genes were found in this region rather than four, since this region was previously thought to have four complementation groups (70). We therefore carried out additional complementation studies, using stable heterodiploid strains rather than the transient heterodiploids used previously. We identified three complementation groups, in agreement with the sequence data. Mutations previously designated as being in the *traH* complementation group were therefore renamed *traI* mutations. It is far from clear why the earlier study indicated that *traI* mutations fell into two complementation groups. However, the sequence of *traI* suggests an internal translation start site at codon 482 (AAGA AGG-N_5-ATG). This region is immediately upstream of the putative helicase domain. This would suggest that TraI could be made in two forms, a full-length form and a truncated form containing only a helicase domain. The TraI protein of F has extremely similar properties, since a protein designated TraI* is translated by using an internal initiation codon. TraI* contains the helicase domain but not the relaxase domain (65). However, if this explanation is correct, is does not explain why only three complementation groups were obtained in the present study. We therefore cannot at this time fully understand the discrepancy between the older analysis and the present one.

We have also described a locus of pKM101 that appears to play a role in preventing recombination-mediated plasmid instability. While the cause of this instability is unclear, several other plasmids have been reported to have functionally similar genes, and in some cases these genes have been shown to encode site-specific recombination systems. These include the *parA* gene of RP4 (12), the D protein of mini-F (29), the *per* gene of R46 (11), and the *cre* gene of bacteriophage P1 (59), among others. The ParA and Per proteins are homologous to the resolvase protein of Tn*3*, while the D and Cre proteins are not homologous to other known proteins, and none is similar to StbA, StbB, or StbC or pKM101. The hallmark of most of these systems is that they are needed only in recombination-proficient hosts. It is believed that two identical copies of any multicopy plasmid can undergo homologous recombination, resulting in a head-to-tail dimer, which would effectively decrease plasmid copy number and could lead to inefficient partitioning to daughter cells during cell division (61). These site-specific recombination systems are thought to convert dimeric plasmids to monomers, thereby enhancing plasmid stability. We postulate that the *stb* locus of pKM101 may play a similar role. If so, it is not clear why three proteins would be required, since all the site-specific recombination systems listed above require just one protein. Perhaps only *stbC* is required for stability, and insertion mutations in *stbA* or *stbB* prevent expression of *stbC* by transcriptional polarity.

Each of these recombinases act at a particular DNA sequence, denoted the *par* site in RP4, the *rfsF* site in mini-F, the *per* site in R46, and the *lox* site in P1. Each of these sites is composed of direct or inverted repeats that provide binding sites for the resolvase proteins. In all cases except the *lox-cre* system, these sites are located directly upstream of the respective recombinase genes, and each recombinase serves as a transcriptional autorepressor as well as a recombinase. This provides a simple mechanism for the synthesis of sufficient

amounts of protein to saturate the binding site. We hypothesize that if in fact the *stb* operon encodes a site-specific recombinase, the direct repeats found directly upstream of *stbA* could provide a cognate resolution site. If so, binding of one or more of these proteins could cause negative autoregulation.

As described above, one of these recombinase systems (*per*) is found on plasmid R46. Interestingly, R46 is the direct parent of pKM101, which was derived by an in vivo deletion of 15 kb of R46 DNA (17, 31). This deleted DNA includes the *per* gene. Therefore, if our model for *stb* function is accurate, it would appear that R46 has two such systems, one encoded by *stb* and the other encoded by *per*. In agreement with previous studies, we find that R46 is not detectably lost from a population of bacteria even after 60 generations (unpublished data). In constrast, pKM101 is lost at a detectable rate, which is consistent with published data that *per* mutants of R46 are detectably unstable (11). In all cases, instability occurred only in a recombination-proficient host.

Plasmid pKM101 (36,255 bp) has now been sequenced in its entirety. Figure 8 shows the positions and transcriptional orientations of all previously characterized genes of this plasmid as well as 10 uncharacterized ORFs. Approximately half of the DNA of this plasmid encodes proteins that play some role in conjugation. pKM101 also contains genes that direct vegetative replication, stable plasmid inheritance, inhibition of host restriction systems, and error-prone repair of damaged DNA (see the legend to Fig. 8 for more detail). A more detailed description of pKM101 and its parent R46 is in preparation and will be the subject of a future study.

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